Wintrobe's CLINICAL HEMATOLOGY

TENTH EDITION

VOLUME 1

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the "traumatic" and "microangiopathic" hemolytic anemias. In these disorders, large red cell fragments are present in the circulating blood. They appear on blood smears as small, misshapen, often triangular or helmet-shaped structures (schistocytes or schizocytes). Fragmentation also occurs within the spleen when the reticulocyte is pitted of red cell inclusions, such as residual organelles and hemosiderin granules, and in Heinz body anemias. Aging red cells become less and less readily deformable (197). It may be that senescent red cells find themselves able to pass through the spleen only with great difficulty and that a small fragment is broken off and ingested by the phagocyte, thus explaining the smaller size and increased density of senescent cells.

That fragmentation is the chief means of destruction of red cells was suggested by Rous and Robertson in 1917 (228). These investigators could find no morphologic signs of disintegrating whole red cells (erythrophagocytosis) in the course of an organ-by-organ search of the body. They therefore concluded that, when the cell is deformed by passage through the microcirculation, portions are broken off until finally a fine, hemoglobin-containing dust is removed by the macrophages of the reticuloendothelial system. As attractive as this hypothesis may be, it fails to explain the absence of dramatically microcytic or deformed cells in the normal circulation, cells that must be present if the aging red cell is truly nibbled away until it vanishes. Furthermore, the hypothesis is most easily comprehensible in terms of the labyrinthine microcirculation of the spleen. In view of the failure of red cell survival to become prolonged following splenectomy, the fragmentation hypothesis probably requires that the hepatic microcirculation is more demanding of the red cell than has been supposed.

Sites of Erythrocyte Destruction

Approximately 80 to 90% of normal erythrocyte destruction occurs without release of hemoglobin into plasma (225,226, 36). Because of this fact, the major part of the destructive process is presumed to be *extravascular*, probably within macrophages of the spleen. Only 10 to 20% of normal destruction occurs *intravascularly*, and this mode of destruction has special characteristics. Most hemolytic anemias are characterized by predominantly extravascular destruction; in some intravascular destruction predominates.

Extravascular Hemolysis

For many years, it was considered that the macrophages of the liver (Kupffer cells) play the major role in the breakdown of red cells. This theory arose because the classic experiments of Minkowsky and Naunyn were conducted in ducks and geese, animals in which hepatic Kupffer cells account for nearly all of the body's macrophages. In humans, fixed macrophages are more widely distributed. It is clear that other organs, especially the spleen, participate in erythrocyte destruction, and that even in contused wounds, or in subcutaneous tissues into which erythrocytes have been injected, erythrocyte breakdown occurs (237).

The relative importance of the spleen and liver in erythrocyte destruction is influenced by the degree of cell damage (223). Severe degrees of damage lead to destruction in all macrophage-containing organs, but especially in the liver because of its relatively great blood flow. The spleen, in contrast, is apparently more sensitive to cell injury; cells only very minimally damaged are preferentially removed by that organ (238). It is probable that effete red cells are destroyed primarily in the spleen; however, if this organ is removed from normal subjects, macrophages found in other organs, especially the liver, rapidly assume this function, and there is no increase in cell survival (239).

The erythroclastic function of the spleen and the mononuclear phagocyte system is discussed in more detail in Chapter 16.

Intravascular Hemolysis

Special features characterize those situations in which red cells are destroyed within the circulation rather than within macrophages. When this happens, hemoglobin is discharged directly into the circulation from which it is removed by several mechanisms (Fig. 12.4).

Haptoglobin (226,240–243,245–247). At low rates of release of hemoglobin into plasma, all of the hemoglobin is found to be attached to haptoglobin. This specific, hemoglobin-binding protein was first detected in plasma by its ability to enhance the peroxidase activity of hemoglobin (248). Because its concentration increases in a variety of inflammatory diseases, an increased haptoglobin level was rec-

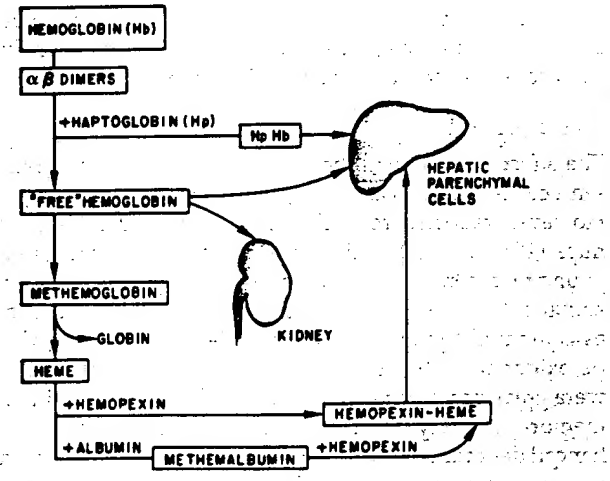

FIG. 12.4. Pathways for the disposal of hemoglobin (Hb) in plasma. Hemoglobin freely dissociates into αβ dimers. These are bound by haptoglobin (Hp) with subsequent removal of the HbHp complex by hepatic parenchymal cells. Hemoglobin in excess of the Hp binding capacity circulates as the unbound (free) protein. In this form it is partially removed by hepatic cells, but it may also follow two other pathways; it may be excreted by the kidney or oxidized to methemoglobin, from which heme is easily dissociated. Heme is initially bound to hemopexin, which transports it to the hepatic parenchymal cell. Heme may also be bound nonspecifically by albumin, forming methemalbumin. This complex probably transfers its heme to hemopexin as the latter becomes available.

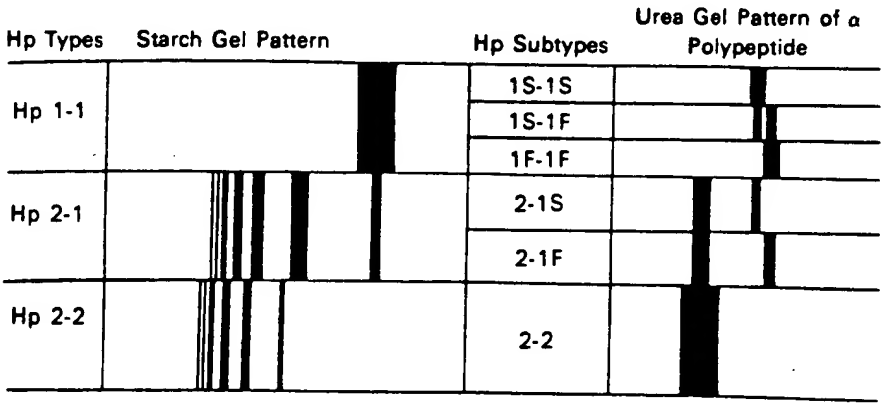

FIG. 12.5. Starch gel electrophoretic patterns of the common haptoglobin (Hp) phenotypes. The three major phenotypes *(left)* can be subdivided on the basis of analysis of the light (α) polypeptide chains *(right)*. (From Giblett ER. Haptoglobin. Vox Sang 1961; 6:513, by permission of the author and Williams & Wilkins.)

ognized as a nonspecific sign of disease with much the same significance as an accelerated sedimentation rate. Later, the role of haptoglobin as a hemoglobin-binding protein and as the principal factor affecting the apparent "renal threshold" for hemoglobin was described (249).

The name refers to a family of α_2-glycoproteins that bind hemoglobin. The tetrameric molecule resembles that of certain immunoglobulins in that it has two light (α) chains and two heavy (β) chains linked in humans by disulfide bonds (250). Haptoglobin is synthesized as a single polypeptide chain that is cleaved post-translationally within the endoplasmic reticulum to generate its α and β subunits (251,252). Transcriptional activity of the haptoglobin gene is promoted by interleukin-1, interleukin-6, and glucocorticoids as a part of the acute phase response to systemic inflammation and related physiologic disturbances (253–257). Strong homologies to the chymotrypsin family of serine proteases have been demonstrated, suggesting an evolutionary relation between haptoglobin and these proteins (258,259); however, haptoglobin has no proteolytic activity. By electrophoresis of serum, several different haptoglobin bands have been demonstrated. Most of the variations are due to differences in the α chains. The observed pattern reflects the genetic constitution of the individual (260). The structural gene for haptoglobin has been localized to the long arm of chromosome 16 (16q22) (261–264). There are three common alleles in humans. Two of these, designated Hp^{1F} and Hp^{1S}, code for electrophoretically distinguishable fast (F) and slow (S) migrating classes of α chains. These polypeptides are of equal length, differing only by the presence of lysine in amino acid position 53 of Hp^{1F} and glutamic acid in Hp^{1S} (258,265,266). The third allele, Hp^2, apparently arose from an intragenic duplication originating from a non-homologous unequal crossover between two Hp^1 alleles (262,267). This crossover was within the fourth intron of an Hp^{1F} allele and the second intron of an Hp^{1S} allele (267). The resulting Hp^2 gene thus has seven exons rather than the five exons present in the Hp^1 alleles (267). Several rarer variants have also been observed on the basis of different electrophoretic patterns (240). In addition, in humans and old world monkeys, the Hp^2 gene is duplicated 2.2 kb downstream. The duplicated gene is referred to as the haptoglobin-related (Hpr) gene. In some blacks, multiple copies are present (267). The genes differ from one another in part because retrovirus-like sequences have been inserted at several sites (267–270). The Hpr gene codes for a protein that is apparently structurally normal (267); the Hpr promoter is active and cell-specific (271), but the gene product has only recently been detected in normal persons (272,273). The protein had earlier been identified in plasma of a pregnant woman at term (274). Its function, if different from products of other haptoglobin genes, is not known. In addition to the Hpr gene, old world monkeys have a third haptoglobin gene downstream from the Hpr locus (275).

Expression of the more common haptoglobin genes described above result in a variety of phenotypes, $Hp^{1F\text{-}1F}$, $Hp^{1F\text{-}1S}$, $Hp^{1S\text{-}1S}$, $Hp^{2\text{-}1F}$, $Hp^{2\text{-}1S}$, and $Hp^{2\text{-}2}$, depending upon the allelic constitution of the individual and therefore upon the α-chain product formed (Fig. 12.5). The geographic distribution of haptoglobin alleles varies widely throughout the world (226,245,276). Variation in the allele frequencies in neighboring populations are often considerable, but rough generalizations are possible. The frequency of Hp^2 is highest in Asia and lowest in Africa. Hp^{1F} is most frequent in Africa and almost absent among Asians and American Indians. Hp^{1S} is most common among North Americans and least common among Africans and Asians. Among whites in the United States, the gene frequencies of Hp^{1F}, Hp^{1S}, and Hp^2 approximate 0.18, 0.17, and 0.66, respectively (266). In American blacks, frequencies of the same alleles were 0.26, 0.29, and 0.53 (225). Supposedly significant departures from expected frequencies of haptoglobin phenotypes and/or genotypes have been observed in patients with an astonishing assortment of clinical conditions, including rheumatoid arthritis (277), sarcoid (278), cirrhosis (279), diabetes mellitus (280), cancer (281), leukemia (282,283), hypertension (284–286), and hypercholesterolemia (287).

The concentration of haptoglobin in plasma has been measured in clinical laboratories by a variety of methods. Early

methods for the determination were based on the hemoglobin-binding capacity of the plasma. Measured in this way, the normal range was 0.4 to 2.0 g Hb/L (225). Most clinical laboratories presently measure haptoglobin directly by radial immunodiffusion or immunonephelometric methods, and phenotyping can be performed employing monoclonal antibodies and immunoblotting (288,289). Normal concentrations differ substantially with technique; ranges such as 0.5 to 1.6 g/L (290) and 0.6 to 3.8 g/L (291) are representative, but each clinical laboratory should establish its own reference values. Values differ somewhat with different haptoglobin phenotypes as well (291,292), but these differences seldom have clinical significance. The concentration is also influenced by age. Ahaptoglobinemia is usual in newborns. Concentrations are measurable by about three months of age and increase gradually to adult levels by age 20 (244,293). Decreased concentrations may also be observed in disorders associated with hemolytic anemia, ineffective erythropoiesis, liver disease, hereditary ahaptoglobinemia, and with pregnancy and estrogen therapy. Increased concentrations may be present in any of those diseases in which concentrations of acute phase reactants are increased, such as infections and malignancies.

Haptoglobin and hemoglobin bind within the vascular compartment in an essentially irreversible, noncovalent complex. The association constant between the two has been estimated to exceed 10^{15} (294). Haptoglobin binds αβ hemoglobin dimers. Thus, dissociation of tetrameric hemoglobin is the necessary rate-limiting step in the reaction (295). The overall stoichiometry for the reaction is one hemoglobin subunit per haptoglobin subunit. There are two independent, non-interacting hemoglobin-binding sites on the haptoglobin molecule (295). Hbα chains are bound with much higher affinity than are β chains (296), with the Hbα chain binding site situated at amino acid residues α121-127 (297). Binding of Hbα chains allosterically induces a high-affinity Hbβ chain-specific binding site (295). The binding sites are situated on the haptoglobin β chain (298), they apparently include lysine 136 and lysine 218 but their precise structures and complete locations are not known (299). The primary region of hemoglobin involved in the intermolecular contact is the $\alpha_1\beta_2$ interface (294). Haptoglobin will complex with oxy-, met-, cyanmet- and carbon monoxyhemoglobin (244), but not with deoxyhemoglobin (295), Hb H ($Hb\beta_4$), Hb Bart's (Hb γ_4) (300), or myoglobin (301). Heme-free globin is bound but heme is not (296).

Haptoglobin is synthesized in the parenchymal cells of the liver (302,303). Synthesis has also been described in lymphocyte cultures (304) and brain (305). When not bound to hemoglobin, it leaves the plasma with a half-disappearance time of 3.5 to 5 days (226,242,306). The HpHb complex leaves much more rapidly, with a half-disappearance time of 9 to 30 minutes. About 50 to 80% of the haptoglobin turnover in the normal subject is accounted for by the rapid pathway (226,242). From the kinetics of hemoglobin turnover, it can be calculated that some 10 to 20% of normal erythrocyte destruction occurs intravascularly and uses the haptoglobin system (225,226). In hemolytic anemias characterized by intravascular hemolysis, catabolism of Hp is so rapid that it essentially disappears from the plasma, a change that is not accompanied by a compensatory increase in haptoglobin synthesis (307). Hypohaptoglobinemia also occurs in hemolytic states associated with predominantly extravascular hemolysis (308,309). The explanation for this observation is not established, but it has been suggested that some hemoglobin may be regurgitated from macrophages when the rate of phagocytosis of erythrocytes or erythrocyte fragments reaches a maximum (225).

Catabolism of the HpHb complex occurs in the hepatic parenchymal cell (306,310). Internalization of the HpHb complex by the hepatocyte is mediated by a receptor specific for the complex (311). The receptor apparently recognizes the conformational change of haptoglobin, brought about by complex formation with hemoglobin (311). The internalized HpHb molecules are found at first in Golgi subfractions but later in more dense cellular fractions associated with unidentified organelles (312,313). The Hb-Hp in these latter fractions is dissociated symmetrically into two 82 kDa subunits, probably a Hb dimer-Hp dimer complex (312,313). The heme moiety is then detached from globin-haptoglobin and apparently bound to another carrier protein before conversion to biliverdin. The globin-Hp complex is thereafter degraded. Haptoglobin is not returned to the plasma.

Because a portion of any plasma hemoglobin not bound to haptoglobin is excreted into the urine, it is reasonable to infer that a normal physiologic function of haptoglobin is to prevent renal loss of hemoglobin, thereby conserving iron and protecting the renal tubular cells from damage (314). Haptoglobin has also been shown to inhibit the lipid peroxidation produced by hemoglobin (315–317). The response of haptoglobin to inflammation as an acute phase reactant suggests an additional function. It is not clear what that role might be, but it has been shown that haptoglobin may modulate lymphocyte responsiveness, including inhibition of the mitogenic response of lymphocytes to phytohemagglutin and concanavalin A (318). The hpr protein, as mentioned previously, has been observed in plasma of pregnant women, and an immunologically indistinguishable protein has been demonstrated histochemically in the cytoplasm of breast cancer cells removed from patients whose malignancy has shown an increased propensity for tumor invasion and early metastasis (274,319). These observations raise the possibility of a related role for haptoglobin in pregnancy and malignancy. These various potential functions of haptoglobin notwithstanding, patients with hereditary ahaptoglobinemia suffer no apparent ill effects.

Hemoglobin and the Kidney (320). The hemoglobin-haptoglobin complex is too large (molecular weight approximately 150 kDa) to pass into the glomerular filtrate. Thus, the level of circulating haptoglobin is the most important determinant of the apparent renal threshold (249). When the haptoglobin is saturated, free (unbound) hemoglobin circulates briefly in plasma. The hepatic parenchymal cell is responsible for the removal of some of the free hemoglobin from plasma (306). In addition, hemoglobin dissociates into αβ dimers which, having a molecular weight of about 32 kDa, readily pass through the glomerulus (321). There is a low (less than 0.6 g/L) renal threshold for free hemoglobin present after

haptoglobin saturation that is related to renal tubular reabsorption (322). The rate of tubular reabsorption of hemoglobin in adult males is 1.43 ± 0.96 mg/min (323). If this capacity is exceeded, hemoglobin appears in the urine. Thus, renal handling of hemoglobin is similar to that of glucose, urate, and certain other substances (321).

Hemoglobinuria, when it is of considerable magnitude, may cause precipitation of heme pigment as casts in the distal tubules, proximal tubule cell necrosis, and acute renal failure. The mechanism is disputed. Several theories have been proposed: (1) that hemoglobin or hemoglobin products are directly toxic to proximal tubule cells; (2) that precipitation of hemoglobin results in tubular obstruction and renal failure; and (3) that direct renal injury by hemoglobin does not occur but instead products of intravascular hemolysis result in hypotension and other systemic and local vascular events, in part perhaps as a result of disseminated intravascular coagulation, and these lead to renal failure (324–327).

Within the tubular epithelial cell, hemoglobin iron is rapidly extracted and stored in the cell as ferritin and hemosiderin (237,321). Some of the tubular epithelial iron may be reused for hemoglobin synthesis, but its mobilization for this purpose occurs only at a very slow rate (321). When iron-laden tubular cells are sloughed into the urine, the urine iron concentration increases and both ferritin and hemosiderin may be detected (328). Clinically, hemosiderinuria is usually detected by the Prussian blue stain of the urinary sediment (329). Detectable hemosiderin usually does not appear in the urine for 48 hours after a specific episode of hemoglobinuria (328) and may persist for more than a week (324). In chronic intravascular hemolysis, such as occurs in red cell fragmentation associated with abnormal prosthetic heart valves, hemosiderinuria is continuous (330) and ultimately may result in iron deficiency.

Plasma Heme, Hemopexin, and Methemalbumin (331–333). Free hemoglobin in plasma is readily oxidized to methemoglobin. The latter dissociates easily and nonenzymatically into heme and globin (334). Free heme is highly insoluble at physiologic pH. However, hemopexin and albumin, and to a lesser degree α-fetoprotein and lipoproteins, are able to bind heme and maintain it in a soluble form. Heme is removed from these proteins by hepatocytes.

Hemopexin is a β1-glycoprotein, consisting of a single polypeptide chain and containing 20% carbohydrate, with a molecular weight of about 70 kDa. It binds heme with the highest known affinity of any heme-binding protein and plays an important role in receptor-mediated hepatocyte heme uptake. The molecule has two similar halves, suggesting duplication of an ancestral gene (335,336). The heme-binding region is not duplicated and may have been formed by conformational interaction between the two homologous halves (332). It has been proposed that the amino acid sequence of hemopexin and porcine liver hyaluronidase are identical (337) but although hemopexin may bind to hyaluron, no hyaluronidase activity has been described (338). There is substantial molecular heterogeneity, presumably the result of variation in the carbohydrate moiety (339,340), but little evidence of genetic polymorphism in most groups studied. However, two additional alleles apparently are expressed in Nigerian and United States blacks (341). The human gene encoding hemopexin has been localized to the region p15.4-p15.5 of chromosome 11 and is thus at the same site as the β-globin gene cluster, genes that also code for polypeptides that share the property of binding heme (342–344).

Hemopexin is synthesized in the liver (345) and is found in the plasma in a concentration of 40 to 150 mg/dl. Each hemopexin molecule avidly binds one molecule of heme (Kd ~10^{-13} M). It also binds other porphyrins and bilirubin but with less avidity (346,347). Binding is through the heme iron to two histidine residues of hemopexin (346,348–353). The half-life of hemopexin in normal subjects is about 7 days (354), whereas the heme-hemopexin complex is removed from the circulation with a half-disappearance time of 7 to 8 hours (331).

Hepatocyte uptake of the heme-hemopexin complex is presumably by receptor-mediated endocytosis. A specific hemopexin receptor in the plasma membrane of the hepatocyte has been reported (355,356). The heme-hemopexin complex remains stable over the pH range of 6.5-10.0, but heme is released at pH 5.0 (349,357). Hemopexin retains its structure at this pH (357). Thus, after endocytosis (357), the two components can dissociate at the acid pH present in the endosome, and the released hemopexin is returned to the plasma as an intact protein (355,357,359,360). Transport of heme within the cytoplasm occurs by means of an intrinsic heme-binding membrane protein (354,355), and iron is rapidly released by heme oxygenase. Interpretation of these data has been questioned (333,361) and a hepatocyte receptor for heme itself has been proposed instead (362,363), with the final delivery of heme to the liver accomplished perhaps by albumin rather than by hemopexin (333). Uptake of the heme-hemopexin complex in vitro is associated with a decrease in membrane transferrin receptors, and induction of heme oxygenase and metallothionein gene expression occurs (364,365). Hemopexin thus serves to conserve iron, and by preventing heme-mediated lipid peroxidation (315), it functions as an antioxidant as well (338,366,367).

Plasma hemopexin values may be reduced following intravascular hemolysis because of increased hemopexin catabolism (354). The depletion is less pronounced than is true of haptoglobin, and low values imply a relatively severe degree of hemolysis. Especially low values are found in thalassemia major and sickle cell anemia (331,354). Hemopexin concentrations may be increased in some chronic neuromuscular diseases and in acute intermittent porphyria in which the rate of synthesis is increased (368,369). Furthermore, hemopexin, like haptoglobin, is an acute phase reactant and plasma levels rise with an inflammatory stimulus (370–372).

Each mole of human albumin can bind several moles of heme (373) to form *methemalbumin* (374). However, only a single primary binding site (Kd = 1×10^{-8}) is present and the secondary binding sites are of low affinity. Albumin from other mammalian species do not all bind heme at all (334). The disappearance of methemalbumin from the circulation is kinetically complex (375), perhaps because of the differences in affinities of the multiple binding sites. Heme added to